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New molecular method for the detection of human papillomavirus type 16 integration

M. P. Cañadas[1,2,*], L. Darwich[1,3,*], G. Sirera[4,5], V. Cirigliano[2], M. Bofill[1,6], B. Clotet[1,5] and S. Videla[4] on behalf of the HIV-HPV Study Group

1) *Retrovirology Laboratory-IrsiCaixa Foundation,* 2) *General Lab, Barcelona,* 3) *Faculty of Veterinary, Department of Sanitat i Anatomia Animal, University Autonomous of Barcelona [UAB],* 4) *Lluita Contra La SIDA Foundation,* 5) *HIV Clinical Unit, Department of Medicine, University Hospital Germans Trias i Pujol, Badalona [Barcelona], UAB and* 6) *Institució Catalana de Recerca i Estudis Avancats [ICREA]*

Abstract

Human papillomavirus (HPV) infection is the cause of cervical cancer. Integration of HPV-16 DNA in cervical cells is considered to be a key event in the progression towards invasive cancer, but little is known about this event in anal carcinogenesis. The integration could be a useful biomarker for cancer progression. Optimized assays are needed to determine the value of real-time detection of HPV integration in longitudinal studies, and this approach is only possible with a high-throughput assay. The aim of this study was to develop a new multiplex real-time PCR assay based on simultaneous amplification of the E2 and E6 HPV open reading frames (ORFs) in order to assess the physical status (episomal and/or integrated) of HPV-16 in anal cells of HIV-positive men. The comparative threshold (Ct) cycle values for E2 and E6 obtained for SiHA cells and artificial mixtures of episomal and integrated DNA were as expected: similar Ct for episomal forms and absence of E2 amplification for integrated forms. The multiplex real-time PCR was tested in 77 consecutive samples from individual HIV-infected patients with HPV-16 anal infection. The integration of HPV-16 was detected in 25 (32%) patients: 23 as mixed (episomal and integrated) and two as completed integrated forms. The integration occurs in the early stage of anal lesions and was associated with the severity of the lesions (p 0.004). The multiplex real-time PCR assay developed in the course of this study was shown to be a simple, sensitive, specific and inexpensive technique which may be applied routinely to detect HPV-16 integration.

Keywords: Anal cytology, HIV men, HPV-16 integration, human papillomavirus, physical status HPV
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Corresponding author and reprint requests: M. P. Cañadas, Department of Molecular Biology, General Lab, Londres 28, 08021Barcelona, Spain
E-mail: bmol@general-lab.es

*Both authors have contributed equally to this study.

Introduction

Human papillomavirus (HPV) infection is well known as the most important cause of cervical cancer, being preceded by precursor lesions that can be classified as low- or high-grade squamous intraepithelial lesions (LSIL or HSIL, respectively). Although these lesions are the result of HPV infection, only a small proportion of infected women will develop cervical cancer. As the clinical outcome of these lesions cannot be predicted on the basis of cytological assessment, different markers have been proposed, e.g. the persistence of the infection and viral integration [1].

The persistent infection of high-risk HPV such as HPV-16 and HPV-18 is directly associated with pre-cancerous lesions. The HPV-16 infection is the most prevalent type found in cervical cancer as well as in anal cancer [2]. HPV infection remains highly prevalent and persistent among HIV-infected patients [3,4] who have an increased risk of developing HPV-related squamous cell cancers in the anus.

The integration of HPV-16 DNA into the host genome is considered an important event in the progression of premalignant cervical lesions to cervical cancer. Early HPV-16 DNA integration in pre-neoplasic cervical lesions has been reported [5–8]. During HPV infection, products of viral E6 and E7 oncoproteins are important for the process of trans-

formation and immortalization of infected cells, causing the development of malignant lesions. These proteins are regulated by the E2 gene product which represses the transcription of the E6 and E7 genes. The E2 open reading frame (ORF) has been identified as the preferential site of HPV integration, being more frequently disrupted or deleted than other sites [9]. Therefore, the lack of control of E2 over E6 and E7 gene transcription, as a consequence of the integration process, could play a role in the progression and development of carcinogenic lesions [7,10]. However, little is known about this phenomenon in anal lesions of HIV-positive patients.

Different methodologies have been used to detect integrated forms, such as Southern blot analysis, two-dimensional gel electrophoresis, amplification by PCR and the recently quantitative real-time PCR [8–11]. Real-time PCR tests are based on the assumption that the episomal (not integrated) form contains the same number of copies of both E2 and E6. When integration occurs, disruption of the E2 gene causes a reduction in the E2 copy number; thus, integration can be detected by measuring the E2/E6 DNA ratio [11]. Although integration could be a good biomarker for carcinogenic progression, the techniques to evaluate this are time consuming. The availability of a simple and sensitive method to detect HPV DNA integration into the human genome would allow routine testing in a clinical setting. The aim of this study was to develop a new single-tube multiplex real-time PCR to analyse the HPV-16 physical status in anal cytological samples of HIV-1 infected men.

Patients and Methods

Study population

Between January 2005 and December 2006, all consecutive samples from individual patients from the CARH-MEN cohort (CAn Ruti HIV-infected MEN) with an HPV-16-positive anal infection were included. The Germans Trias i Pujol Hospital ethical committee approved the protocol and written informed consent was obtained from all participants enrolled in the study.

New multiplex real-time PCR (TaqMan assay)

To study the physical status of HPV-16, a new multiplex real-time PCR method was developed for simultaneous amplification of the E2 and E6 regions. This method is based on the assumption that when the virus is integrated, the E2 region is deleted, allowing the detection of integration as a reduced degree, or total absence, of E2 amplification. PCR primers and minor groove binder probes were designed using Primers Express software (Applied Biosystems, Foster City, CA, USA); the sequences and fluorochromes are shown in Table 1. Amplification was optimized to be performed in 25 μL, including DNA in 5 μL, 2× Universal Real-time Master mix (Applied Biosystems), 30 pmol of E2 forward and reverse primers, 6 pmol of E2 probe, 3 pmol of E6 primers and 1 pmol of E6 probe. PCR was carried out for 45 repeat cycles at 50°C for 2 min, 95°C for 1 min, 55°C for 1 min and 72°C for 1 min in a 7300 PCR systems apparatus (Applied Biosystems).

Different physical states (episomal, integrated or both) were identified by comparing the threshold cycle (Ct) between E2 and E6. The same Ct was expected for episomal forms (same amount of E6 and E2 products), an increased E2 Ct for mixed (episomal and integrated) forms, and an undetectable E2 Ct for integrated forms.

Control samples of HPV-DNA integration. SiHA cervical carcinoma cells were used as control for HPV-DNA integration. SiHA cell lines have one–two copies of integrated HPV-16 in which the E2 gene is deleted and the E6 is retained [12]. DNA extracted from four anal cell samples obtained from asymptomatic patients with transient HPV-16 infection (defined as HPV-16− positive that were found negative for HPV infection after two consecutive visits) was considered to have only episomal HPV and was used as episomal con-

TABLE 1. Primer and probe sequences used for real-time PCR

Primer pair	Primer sequence[a]	Primer location[b]	Size (bp)
E2F	5′-GCAACGAAGTATCCTCTCCTGAA-3′	3358–3381	82
E2R	5′-AAGGCGACGGCTTTGGTAT-3′	3419–3440	
E6F	5′-ACCGGTTAGTATAAAAGCAGACATTTTAT-3′	55–84	70
E6R	5′-GCTCCTGTGGGTCCTGAAAC-3′	105–125	
Probe			
E2-Probe	TTATTAGGCAGCACTTGGC-FAM	3383–3401	
E6-Probe	CACCAAAAGAGAACTGC-VIC	86–102	

[a]Primers and probes were designed based on sequences available for the HPV-16 reference strain (Gen Bank accession number NC001526).
[b]Numbers correspond to positions within the HPV-16 reference sequence; bp, base pairs.

trols. Episomal HPV-16 in samples was quantified using a standard calibration curve constructed by amplifying E6 DNA from SiHA cells at known concentrations obtained after serial dilutions (1×10^4 to 1×10^1 copies). Artificial mixtures, prepared by adding different SiHa DNA amounts (*c.* 1×10^4, 1×10^3, 1×10^2, and 1×10 copies) to quantified episomal samples, were used for real-time PCR development to simulate the environment existing in clinical samples.

Cytological diagnosis of anal samples. Anal samples were obtained with a brush and kept in PreservCyt/ThinPrep solutions. The cytological changes were classified according to the Bethesda System: normal, ASCUS (atypical squamous cells of uncertain significance), LSIL or HSIL. All samples were analysed by two cytopathologists.

HPV detection and typing. After cytological analysis, DNA was extracted from cellular suspensions using the Qiamp Viral DNA kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Samples were stored at −40°C until analysis. HPV detection and typing were performed in all samples using the *F*-HPV typing kit (Molgentix, Spain) in accordance with the manufacturer's instructions. Briefly, extracted DNA was amplified using a multiplex *F*-HPV PCR with a set of 15 fluorochrome-labelled primers recognizing HPV types within the E6 and E7 regions of the HPV genome. The *F*-HPV amplification was performed in a final PCR volume of 25 μL containing 20 μL of reaction mixture and 5 μL of extracted DNA for 35 repeat cycles of 30 s at 95°C, 30 s at 64°C and 30 s at 72°C. Products were analysed using capillary electrophoresis on an ABI 3130 XL genetic analyser and GeneMapper 4.0 Software (Applied Biosystems).

Statistical analyses

A descriptive analysis was performed. Prevalence and corresponding 95% confidence intervals (95% CI) were calculated for anal HPV-infection, anal HPV-16 infection and anal cytological lesions.

Fisher's exact test was used to assess associations between the physical status of HPV-16 and the degree of anal pathology. Repeatability and reproducibility of the real-time PCR assay were assessed by calculating the coefficient of variation (CVs) of the increment of Ct (ΔCt) value. A p value ≤ 0.05 was considered statistically significant. All data were recorded in a database program (Microsoft Access for Windows XP, Redmont, CA, USA). Data analysis was performed using the statistical software program SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Optimization of multiplex real-time PCR

The multiplex real-time PCR set up was based on titrating different concentrations of primers and probes to obtain the expected relative Ct values for the different physical forms. Similar Ct values were obtained for E6 and E2 in episomal forms (Fig. 1a). As expected, no E2 amplification was detected in completely integrated forms (Fig. 1b). The amplification efficiency of E2 and E6 with different input DNAs was also assessed in serial dilutions of episomal samples. Using the artificial mix with SIHA cell lines (mixed episomal/integrated samples), the E2 Ct values increased with regard to E6 so that a ΔCt could be calculated (Fig. 1c).

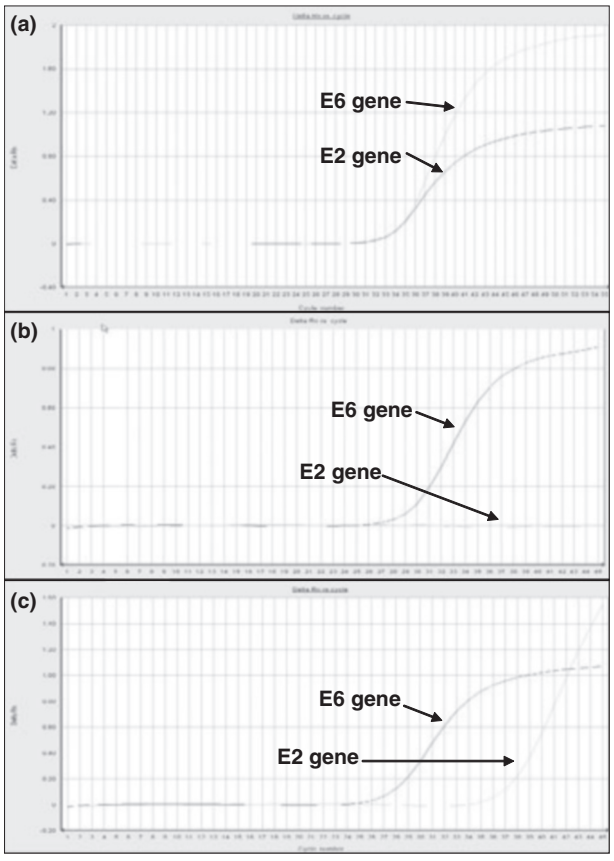

FIG. 1. (a) Real-time PCR detection of HPV-16 episomal forms in clinical samples. E2 and E6 are present in the same amount and amplified with the same comparative threshold (Ct). (b) Detection of the fully integrated form. The E2 amplicon is disrupted by integration and only E6 is amplified. (c) Detection of mixed (episomal and integrated) forms. As result of partial integration, E2 copies decrease relative to E6, thus producing a difference in Ct [ΔCt].

TABLE 2. Variation in ΔCt, repeatability and reproducibility of the *Taq*Man assay for integration of HPV-16

	ΔCt E6E2 (mean ± SD)	
ID	**Intra-assay**	**Inter-assay**
Sample A	0.73 ± 0.22	0.57 ± 0.2
Sample B	0.32 ± 0.15	0.34 ± 0.1
Sample C	0.44 ± 0.24	0.28 ± 0.23
Sample D	0.19 ± 0.15	0.2 ± 0.16

Values are means ± standard deviation (SD) of ΔCt (differences in Ct values of E2 with regard to E6). The values are based on independent measurements of episomal samples by testing four cases in triplicate on different days.
Values up to 1 are considered to indicate integrated forms.

The variability of E2 and E6 ΔCt in episomal samples was evaluated by testing four cases in triplicate on different days; this allowed us to assess the repeatability and reproducibility of the assay (Table 2). Based on the values of ΔCt (max. 0.73 ± 0.22, min. 0.19 ± 0.15), samples with ΔCt values of up to 1 were considered to be a mixed form.

The sensitivity of this method in discriminating different HPV physical forms was assessed in artificial mixtures of SiHa DNA in episomal DNA samples at known concentrations, tested in triplicate on three different days (Table 3). The assay was found to be sensitive enough to detect integrated forms even if present at a concentration five times lower than episomal forms. This ratio was found to be maintained at concentrations of episomal forms between 5000 and 10 copies (23 and 32 Cts, respectively), but at higher concentrations of episomal forms (i.e. 50 000 copies), 1:5 integration was not sufficient to be detected with the assay.

Population used to test multiplex real-time PCR

HIV-infected patients (n = 269) were visited and tested for anal HPV-16 infection which was found in 77 of them (29%, 95% CI: 23–34%). The same samples were used to assess HPV-16 integration. In this group of HIV-infected patients with anal HPV-16 infection, 77% (59/77, 95% CI: 66–86%) had anal cytological abnormalities with the following cytological diagnoses: ASCUS 22% (17/77), LSIL 41% (32/77) and HSIL 13% (10/77). Eighteen (23%) patients had normal cytological results.

HPV-16 integration status

Integration was detected in 32% (25/77, 95%CI: 22–44%) of HPV-16-positive samples. In 23 of these cases, integration was found to be mixed (episomal and integrated) whereas in two cases full integration was observed. It is important to point out that the overall HPV-16 integration rate was 9% (25/269, 95% CI: 6–13%).

The 18% (3/17) of samples with integrated HPV-16 forms were diagnosed as ASCUS, 44% (14/32) as LSIL, 70% (7/10) as HSIL and only one (6%, 1/18) of these integrated samples had a normal cytology (Fig. 2). The presence of integrated HPV-16 forms in cytological samples was associated with the severity of anal lesions ($p < 0.05$).

TABLE 3. Assay sensitivity in discriminating HPV physical forms assessed in artificial mixtures of SiHa DNA in episomal DNA samples at known concentrations

			ΔCt E6E2		
ID sample	**EPISOMAL (mean copies)**	**INTEGRATED (mean SiHA copies)**	**1st RUN**	**2nd RUN**	**3rd RUN**
1	50 000	10 000	0.62	0.26	0.43
	50 000	1000	0.79	0.37	0.11
	50 000	100	0.34	0.2	0.08
	50 000	10	0.53	0.18	0.05
2	5000	10 000	1.21	2.34	3.05
	5000	**1000**	**1.05**	**1.13**	**1.26**
	5000	100	0.43	0.47	0.64
	5000	10	0.32	0.17	0.31
3	500	10 000	6.01	7.16	5
	500	1000	2.72	5.36	2.5
	500	**100**	**1.21**	**1.1**	**1.16**
	500	10	0.32	0.21	0.75
4	100	10 000	7.3	8	8.2
	100	1000	3.7	4.34	5
	100	**100**	**1.12**	**1.19**	**1.8**
	100	10	0.42	0.23	ND
5	10	10 000	8.42	9.98	ND
	10	1000	9	8.01	ND
	10	100	4.1	4.03	ND
	10	**10**	**1.04**	**0.96**	**ND**

ND, not done.
ΔCts (differences in E2 Ct values with regard to E6) obtained in independent measurements of artificial mixtures of SiHa DNA in episomal DNA samples at known concentrations, tested in triplicate on three different days.
In bold are relative concentrations where integration is clearly detectable with ΔCt values of at least 1.

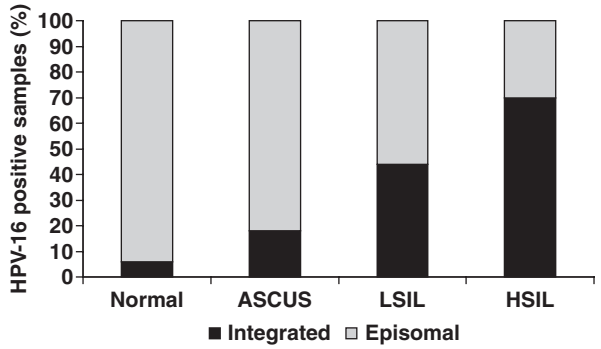

FIG. 2. Frequency distribution of HPV-16 DNA physical state in anal cytological samples kept in PreservCyt/ThinPrep solutions. ASCUS, atypical squamous cells of uncertain significance ($n = 17$); LSIL, low-grade squamous intraepithelial lesions ($n = 32$); HSIL, high-grade squamous intraepithelial lesions ($n = 10$).

Discussion

This is a report of the most extensive study of HPV-16 integration prevalence, by means of a new multiplex real-time PCR, in anal cell samples of HIV-1-infected men with no previous history of anal pathology.

Given that integration could be a useful biomarker for cancer progression, optimized assays are needed to determine the value of real-time detection of HPV integration in longitudinal studies. This approach is only possible with a high-throughput assay that allows the analysis of large sample sizes. In this study, a new and sensitive real-time PCR method was used to analyse integration, although other real-time PCR methods have recently been used in some studies [10,13–18]. All methods used up to now have been based on two separate real-time PCR quantifications of HPV-16 E2 and E6 genes. In previous real-time PCR methods, total viral load was assessed by E6 quantification and the integration was calculated by quantifying the E2 gene in the same sample and comparing the different amounts determined. A cut-off based on the E2/E6 ratio values was then established to assess the status of episomal, integrated or mixed forms. However, these procedures presented several drawbacks [9,11–20]. First, different cut-off values have been used, producing mixed and ambiguous results. Second, the PCR measurement of two independent sequences amplified in different reactions can be hampered by several factors, e.g. the relative concentration of the initial targets, the different PCR efficiencies with different primer pairs, and human pipetting errors that, even if small, could greatly alter the final results. In order to minimize these limitations, common strategies were carried out: (i) accurately measuring the DNA amount used as PCR input, (ii) running samples in duplicate or triplicate and, finally, (iii) assessing cut-off values between separately quantified sequences. Despite these approaches, real-time PCR analysis of HPV integrations produced non-reproducible results.

Ideally, the most accurate way to quantify two viral genes independently from their relative amounts in the initial template and from possible run-to-run variation as a result of human error would be the simultaneous amplification in the same PCR followed by a relative quantification. In this way, possible changes affecting PCR efficiency would have the same effect on both sequences, thus not influencing the final result. We developed a new single-tube multiplex real-time PCR that allowed simple and sensitive detection of integrated forms without the need for prior accurate DNA quantification. HPV integration is easily detected as an increase in the E2 Ct value if compared with E6 amplified in the same PCR.

Spiking experiments with different proportions of SiHa cells in episomal samples also allowed us to assess the suitability of the assay to discriminate mixed from episomal forms even if integration was present in only one-fifth of the total template, which is an improvement over previously reported data [21].

The prevalence of integration detected in normal anal cytological analysis is lower (6%, one positive sample out of 18) than previously observed in cervical samples, where over 50% of cases with normal cytology have been reported with HPV integration [13,15,16]. These data also raised concerns about the clinical significance of HPV integration being observed in such a high proportion of normal samples [21]. Nevertheless, a recent re-analysis of cut-off values has been introduced and changes in interpretation of the results may suggest an overestimation of integrated forms in a high proportion of cases, which ideally should lead to a revision of previously reported data [22]. On the other hand, given that a unique positive result of integration in a sample with normal cytological diagnosis could represent a false-negative cytological result, our results are in agreement with data reported on cervical samples that were tested using alternative molecular methods such as amplification of papillomavirus oncogne transcripts or Southern blotting where no integration has been detected in samples with normal cytology [6,9].

The prevalence of integration (32%) was similar to that found in the only available study reporting 25% integrated forms in anal lesions from men in a small subset of 16 patients [21]. Interestingly, in the same study, a strong correlation between HPV-16 status and the presence of chromosome copy-number abnormalities (CNAs) has also been reported, confirming the hypothesis that integration may lead to increased chromosomal instability as a result of the loss of E2-mediated repression of E6 and E7 expression. In fact,

we have observed HPV integration in only 70% of high grade anal lesions. This may be because of viral DNA disruption outside the E2 region targeted by our assay. As a consequence, the prevalence of HPV-16 integration could be underestimated. Recently, this has been shown to be the most likely sequence to be disrupted during integration [20]. Therefore, alternative mechanisms to HPV integration (methylation in host cells or in the HPV genome, environmental factors and pharmacological treatments) could directly affect gene expression leading, to DNA instability and finally to cancer progression.

Longitudinal studies with large sample sizes are necessary to determine the importance of the physical status of HPV-16 as a predictive marker for cancer progression, and this approach will only be possible with a high-throughput method. Moreover, high-resolution anoscopy has been shown to be a more sensitive tool for diagnosing high-grade anal neoplasia [23]. In this context, HPV-16 integration should be tested in anal biopsies. The multiplex real-time PCR assay developed in the course of this study was shown to be a simple, sensitive, specific and inexpensive technique which allows the detection of forms of HPV-16 integration using routinely collected samples.

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HIV-HPV Study Group: University Hospital Germans Trias i Pujol, Badalona [Barcelona]. University Autonomous of Barcelona: Department of Proctology: M. Piñol, F. García-Cuyas; Department of Pathology: E. Castella, M. Llatjós; HIV Clinical Unit and Internal Medicine Department: C. Rey-Joly, A. Bonjoch, M. Jabaloyas, T. Jou, J. Moltó, E. Negredo, J. Romeu, C. Tural; Nurses of Proctology Unit: P. Cobarsi, I. Fernández; General Lab, Department of Molecular Biology: L. Rueda, E. Ordoñez.

Transparency Declaration

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